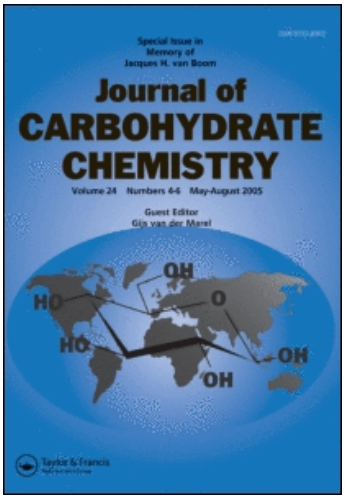

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SYNTHESIS OF THE TRISACCHARIDE α-L-RHA-(1-2)-α-L-RHA-(1-2)-α-L-RHA WITH A DIOXOLANE-TYPE SPACER-ARM[1]

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ABSTRACT

The trisaccharide α-L-Rha-(1-2)-α-L-Rha-(1-2)-α-L-Rha with a dioxolane-type spacer was obtained by using the trichloroacetimidate method in all of the glycosidation steps. After deprotection, the trisaccharide was coupled to BSA or KLH by reductive amination of the spacer aldehyde group.

INTRODUCTION

Group B streptococci (GBS) are a leading cause of neonatal sepsis and meningitis.[2,3] They have been differentiated from other members of the genus by extraction and serologic detection of the group-specific common antigen polysaccharide ("C-substance").[4] The group B antigen has a very complex highly branched structure[5] that was demonstrated recently to be composed of several oligosaccharides linked by phosphodiester bonds.[5,6] However, rabbit polyclonal antibodies that are currently used for immunodiagnosis recognize only the terminal rhamnose-containing trisaccharide: Rha-α-(1-2)-

Rha-α-(1-2)-Rha-α.[7,8] A clinical need exists for rapid detection of the pathogen in neonatal sepsis, and especially for maternal carriers as the vast majority of cases results from vertical transmission.[9]

As part of a project devoted to improving the existing methodologies for the diagnosis of infection by Group B streptococcus, we prepared the synthetic trisaccharide provided with a dioxolane-type spacer arm and coupled the element to different carrier proteins.

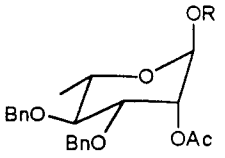
RESULTS AND DISCUSSION

Previous syntheses of Rha-α-(1-2)-Rha-α-(1-2)-Rha α-glycoside bearing methyl[11] or 1-*O*-D-glucitol[8] as aglycon have been reported, using benzyl groups as a permanent protecting group for OH-3 and -4 and an acetyl group at OH-2 which serves in a dual functional role, i.e., as a temporary protecting group and as a participating group.

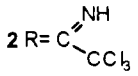
1,2-Di-*O*-acetyl-3,4-di-*O*-benzyl-α-L-rhamnopyranose **1** is the more simple donor in this series, and an intermediate for the synthesis of the other donors. We developed an improved procedure for the preparation of crystalline **1** in a yield exceeding 90 %, starting from acetobromorhamnose through the orthoester that was finally acetolyzed.

The reaction of **1** with 5-(1,3-dioxolan-2-yl)-3-oxabutanol[12] required a high concentration of trimethylsilyl triflate, affording the corresponding rhamnoside **3** in a low yield. In a search for a more potent donor, the diacetate **1** was transformed into the imidate **2** (78 %) by regioselective deacetylation with ethanolamine in ethyl acetate[13] followed by imidation (CCl_3CN-K_2CO_3). This imidate reacts smoothly with 5-(1,3-dioxolan-2-yl)-3-oxabutanol at a ten times lower triflate concentration to afford **3** in an excellent yield (94%). The ^1H NMR spectra of **3** and all the derivatives containing the spacer shows a typical triplet at δ 5.05 and in the ^{13}C NMR spectra signals at δ 102.4 and 65.0 are observed.

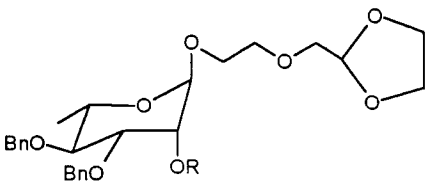
Deacetylation of **3** (→ **4**) and subsequent rhamnosylation with **2** afforded disaccharide **5** in 79 % yield. Further deacetylation (→ **6**) and rhamnosylation with **2** gave trisaccharide **7** in 76 % yield. The structure of **7** was confirmed by the presence in the ano-



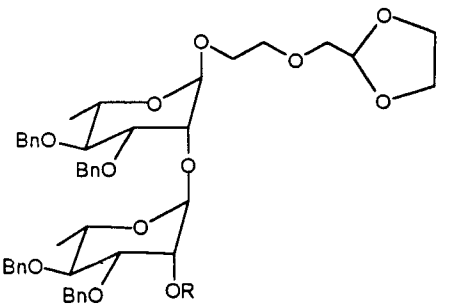
1 R= OAc



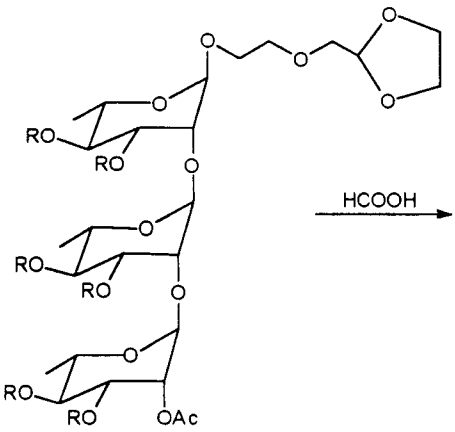
2 R= C(=NH)CCl$_3$



3 R=Ac
4 R= H

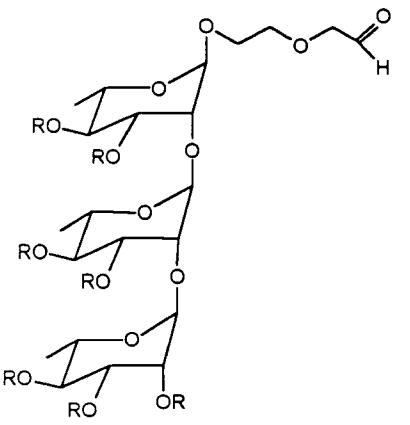


5 R= Ac
6 R=H



7 R= Bn
8 R= Ac

HCOOH



9 R= Ac
10 R= H

meric region of the ^{13}C NMR spectrum of 4 signals corresponding to dioxolan at δ 102.6 and to C-1, -1', and -1" at δ 100.3, 99.1, and 98.9, respectively.

After hydrogenolysis and acetylation of **7**, trisaccharide **8**, obtained in a yield of 96 % was further studied by H-H and X-H correlation spectroscopy, as shown the Figure. The signals corresponding to H-1 and H-1' were obviously correlated with H-2 and H-2', C-1 and C-1'. C-2 and C-2' were deshielded as expected from the α-effect of rhamnosylation. H-1" correlated with C-1", and H-2"(downfield) with C-2"(upfield), according to the presence of an acetyl group at OH-2".

The dioxolane group in **8** was split off by treatment with formic acid for 2 h at room temperature, yielding **9**. Careful deacetylation of **9** with 0.1 M sodium methoxide in methanol at 0 °C afforded the free trisaccharide **10** with an aldehyde group in the spacer. The coupling with BSA or KLH using reductive amination[13] gave neoglycoproteins containing 20 and 336 mol of oligosaccharide per mol of protein, respectively. The use of these neoglycoproteins for the preparation of monoclonal antibodies is now being explored.

EXPERIMENTAL

General methods. Optical rotations were measured at 25 °C with a POLAMAT A automatic polarimeter, using a 5-cm 5-mL cell. NMR spectra were recorded at 25 °C with a BRUKER AC-250F spectrometer. ^{1}H and ^{13}C assignments were made on the basis of homo- and heteronuclear correlation experiments. Chemical shifts (δ) are given in ppm relative to the signal for internal tetramethylsilane; indirectly to $CDCl_3$, δ 77.03, for ^{13}C.

All compounds characterized were purified by column chromatography on Kieselgel 60 (Fluka, < 230 mesh ASTM) and fractions were monitored by TLC on kieselgel 60 F_{254} (Merck). Detection was effected by charring with sulfuric acid after examination under UV light. Evaporations were conducted under reduced pressure at 50 °C (bath).

1,2-Di-*O*-acetyl-3,4-di-*O*-benzyl-α-L-rhamnopyranose (1). A solution of acetobromorhamnose (6 g, 19.6 mmol), methanol (1.7 mL), 2,4,6-trimethylpyridine (2.32 mL, 17.6 mmol) and tetraethylammonium bromide (1.4 g, 6.6 mmol) in dichloromethane (27.6 mL) was refluxed for 1 h. After cooling, the solution was washed with water, dried, filtered and concentrated. To a solution of the resulting syrup in methanol (300 mL) was

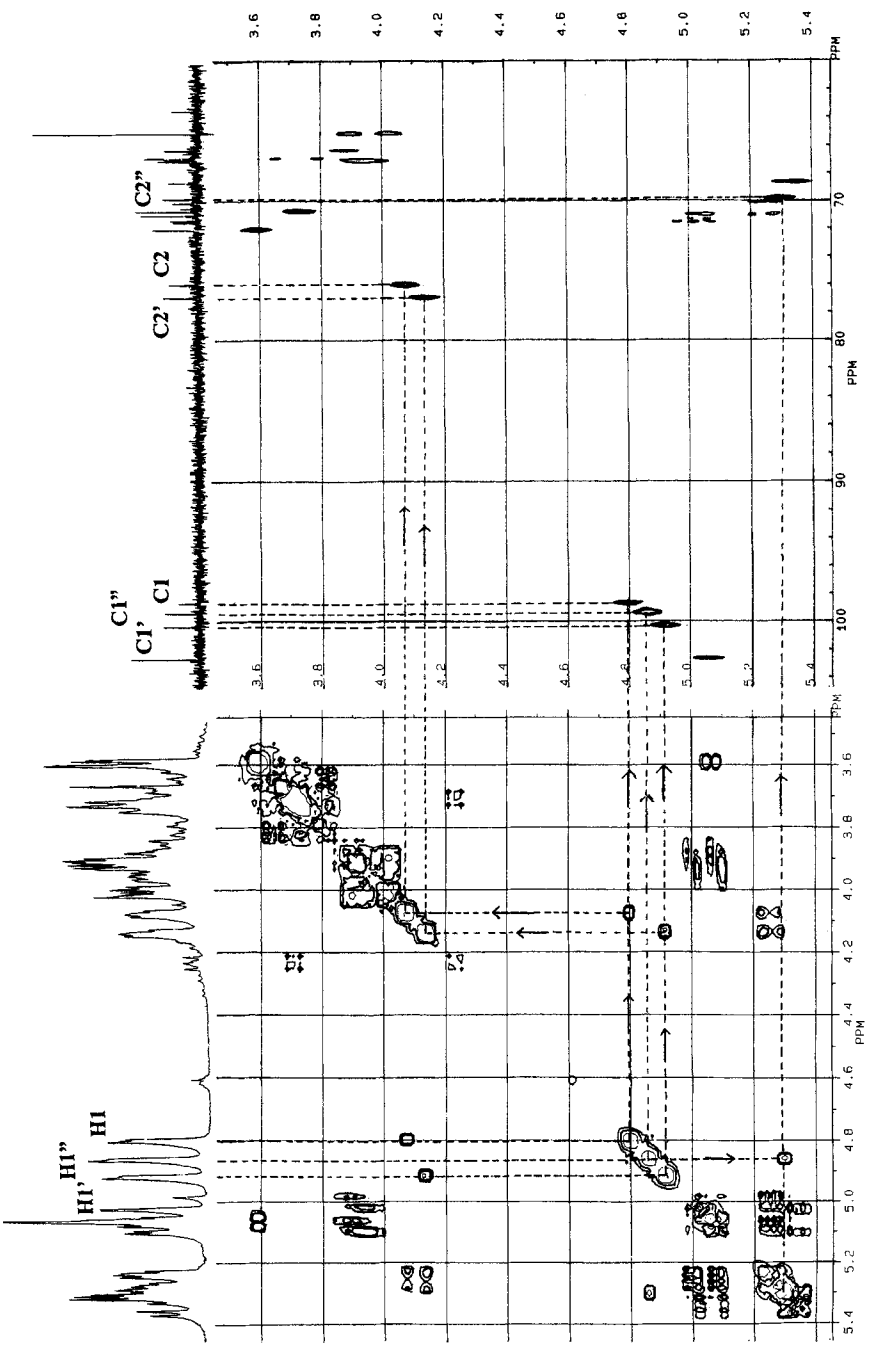


Figure H - H and X - H correlation NMR spectrum of trisaccharide 8

added sodium until the pH attained 9. After 2 h, the mixture was neutralized with Dowex 50 H^+, filtered, concentrated, and dried *in vacuo*. To a solution of the residue in *N,N'*-dimethylformamide (50 mL), cooled to 0 °C, was added sodium hydride (1.2 g, 48 mmol) in small portions. After 30 min, benzyl chloride (8 mL, 32 mmol) was added at $t < 5$ °C, and the mixture was stirred for 12 h at ambient temperature. When TLC (ethyl acetate/hexane 3:1 v/v) revealed complete conversion of the starting material, methanol was added, and the mixture was concentrated. The residue was dissolved in acetic acid/acetic anhydride (2:1 v/v, 30 mL) and cooled to 0 °C. Then, a solution of sulfuric acid (0.25 mL) in acetic anhydride (25 mL) was slowly added. After 20 min, the mixture was poured into stirred ice-cold water. After 1 h, the aqueous solution was discarded and the semi-solid material was washed several times with water. Recrystallization from ethanol afforded **1** (5.6 g, 94 %): mp 109-110 °C; lit[9, 10] mp 107-109 °C; $[\alpha]_D$ -20 ° (*c* 1.0, chloroform); R_F 0.65 (ethyl acetate/hexane 3:1 v/v); ^{1}H NMR ($CDCl_3$) δ 7.4 (m, 10H, 2 Ph), 6.00 (d, 1H, $J_{1,2}$ = 2.1 Hz, H-1), 5.35 (dd, 1H, $J_{2,3}$ = 3.3 Hz, H-2), 4.50-5.00 (2dd, 4H, 2PhCH_2), 3.95 (dd, 1H, $J_{3,4}$ = 10 Hz, H-3), 3.80 (m, 1H, H-5), 3.50 (t, 1H, $J_{4,5}$ = 10 Hz), 2.10 and 2.20 (2s, 6H, 2 Ac), 1.35 (d, 3H, H-6); ^{13}C NMR ($CDCl_3$) δ 170.0 and 168.5 (C=O), 137.7, 138.1 and 127.8-128.4 (Ph), 91.1 (C-1), 77.5 and 79.4 (C-3,4), 71.9 and 75.8 (PhCH_2), 70.0 (C-2), 67.7 (C-5), 20.9 and 20.9 (CH_3CO), 17.9 (C-6).

2-*O*-Acetyl-3,4-di-*O*-benzyl-α-L-rhamnopyranosyl Trichloroacetimidate (2). A solution of **1** (1 g, 2.3 mmol) and ethanolamine (0.39 mL, 4.6 mmol) in ethyl acetate (5 mL) was stirred at ambient temperature. After 16 h, TLC (hexane/ethyl acetate 2:1 v/v) revealed a complete conversion of the starting material into a more polar compound (R_F 0.25). The reaction mixture was filtered over a path of silicagel and the filtrate was concentrated to dryness. A solution of the resulting syrup, potassium carbonate (0.33 g) and trichloroacetonitrile (1 mL) in dichloromethane (10 mL) was stirred for 12 h. Diethyl ether was added and the mixture was filtered over a path of Celite and concentrated to yield chromatographically homogeneous **2** (850 mg, 78 %): R_F 0.55 (hexane/ethyl acetate 2:1 v/v); ^{1}H NMR ($CDCl_3$) δ 8.65 (s, 1H, NH), 6.17 (d, 1H, $J_{1,2}$ = 1.9 Hz, H-1), 5.48 (dd, 1H, $J_{2,3}$ = 3.3 Hz, H-2), 4.76 (dd, 2H, PhCH_2), 4.68 (dd, 2H, PhCH_2), 3.99 (dd, 1H, $J_{3,4}$ = 9.5 Hz, H-3), 3.96 (m, 1H, H-5), 3.49 (t, 1H, $J_{4,5}$ = 9.5 Hz, H-4), 2.20 (s, 3H, Ac), 1.32 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6); ^{13}C NMR ($CDCl_3$) δ 170.0 (C=O), 160.0 (C=NH), 137.5,

137.5 and 127.8-128.4 (Ph), 95.1 (C-1), 79.3 (C-4), 77.1 (C-3), 72.0 and 75.6 (2PhCH$_2$), 70.7 (C-2), 67.5 (C-5), 21.0 (CH_3CO), 17.9 (C-6).

5-(1,3-Dioxolan-2-yl)-3-oxabutyl 2-*O*-Acetyl-3,4-di-*O*-benzyl-α-L-rhamnopyranoside (3). To a solution of **2** (100 mg, 0.180 mmol), 5-(1,3-dioxolan-2-yl)-3-oxabutanol (40 mg, 0.270 mmol) and molecular sieves 0.4 nm in dichloromethane (4 mL) was injected trimethylsilyl triflate (11.7 μL, 0.054 mmol) under argon atmosphere. After 20 min, TLC (hexane/ethyl acetate 1:1 v/v) indicated the disappearance of the starting material (R_F 0.70) and a new spot at R_F 0.40. The mixture was diluted with dichloromethane, treated with triethylamine, filtered, and concentrated. Column chromatography (hexane/ethyl acetate 1:1 v/v) of the residue afforded **3** (115 mg, 94 %) as a syrup: $[\alpha]_D$ = -11° (*c* 1.0, chloroform); R_F 0.40 (hexane/ethyl acetate 1:1 v/v); ^{1}H NMR ($CDCl_3$) δ 5.41 (dd, 1H, H-2), 5.05 (t, 1H, dioxolan), 4.75 (d, 1H, H-1), 4.75 (dd, 2H, PhCH_2), 4.67 (dd, 2H, PhCH_2), 3.92 (dd, 1H, H-3), 3.45 (t, 1H, H-4), 3.80 (m, 1H, H-5), 1.32 (d, 3H, H-6), 2.15 (s, Ac).

5-(1,3-Dioxolan-2-yl)-3-oxabutyl 3,4-Di-*O*-benzyl-α-L-rhamnopyranoside (4). Compound **4** was obtained quantitatively by deacetylation of **3** with sodium methoxide in dry methanol: $[\alpha]_D$ -18° (*c* 1.0, chloroform); ^{1}H NMR ($CDCl_3$) δ 5.01 (t, 1H, dioxolan), 4.82 (d, 1H, H-1), 4.75 (dd, 2H, PhCH_2), 4.67 (dd, 2H, PhCH_2), 4.05 (dd, 1H, H-2), 3.85 (dd, 1H, H-3), 3.45 (t, 1H, H-4), 3.75 (m, 1H, H-5), 1.30 (d, 3H, H-6); ^{13}C NMR ($CDCl_3$) δ 102.5 (C-dioxolan), 99.0 (C-1), 79.8 (C-3), 79.8 (C-4), 71.9, 71.8 and 70.6 (spacer), 68.3 (C-2), 67.3 (C-5), 17.8 (C-6).

Anal. Calcd for $C_{26}H_{34}O_8$: C, 65.80; H, 7.22. Found: C, 65.80; H, 7.44.

5-(1,3-Dioxolan-2-yl)-3-oxabutyl 3,4-Di-*O*-benzyl-2-*O*-(2-*O*-acetyl-3,4-di-*O*-benzyl-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (5). A mixture of donor **2** (100 mg, 0.180 mmol) and acceptor **4** (77.5 mg, 0.163 mmol) in anhydrous dichloromethane (6 mL) was stirred in the presence of molecular sieves 0.4 nm for 20 min under a nitrogen atmosphere. Trimethylsilyl triflate (12 μL, 0.056 mmol) was added and the mixture was stirred for 30 min. Then, TLC (hexane/ethyl acetate 1:1 v/v) indicated an almost complete conversion of **2** (R_F 0.70) and **4** (R_F 0.41) into a compound with R_F 0.46. The reaction was quenched with triethylamine, filtered, and concentrated. Column chromatography (hexane/ethyl acetate 1:1 v/v) of the residue afforded **5** (139.6 mg, 79 %) as a syrup: $[\alpha]_D$

-9° (*c* 1.0, chloroform); R_F 0.46 (hexane/ethyl acetate 1:1 v/v); ^{1}H NMR ($CDCl_3$) δ 5.54 (dd, 1H, H-2'), 5.03 (t, 1H, dioxolan), 4.98 (d, 1H, H-1'), 4.88 (dd, 1H, H-2), 4.73 (d, 1H, H-1), 3.41 (2t, 2H, H-4,4'), 2.11 (s, 3H, Ac), 1.31 (2d, 6H, H-6,6'); ^{13}C NMR ($CDCl_3$) δ 170.0 (C=O), 102.7 (C-dioxolan), 99.2 (C-1), 98.8 (C-1'), 22.0 (CH_3CO), 18.0 (C-6,6').

Anal. Calcd for $C_{48}H_{58}O_{13}$: C, 68.39; H, 6.93. Found: C, 68.36; H, 7.30.

5-(1,3-Dioxolan-2-yl)-3-oxabutyl 3,4-Di-*O*-benzyl-2-*O*-[3,4-di-*O*-benzyl-2-*O*-(2-*O*-acetyl-3,4-di-*O*-benzyl-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl]-α-L-rhamnopyranoside (7). Deacetylation of disaccharide **5** afforded the acceptor **6** which was used directly. A mixture of donor **2** (100 mg, 0.180 mmol) and acceptor **6** (130 mg, 0.163 mmol) in anhydrous dichloromethane (6 mL) was stirred in the presence of molecular sieves 0.4 nm for 20 min under a nitrogen atmosphere. Trimethylsilyl triflate (12 μL, 0.056 mmol) was added and the mixture was stirred for 30 min. TLC (hexane/ethyl acetate 1:1 v/v) indicated an almost complete conversion of **2** (R_F 0.70) and **6** (R_F 0.46) into a compound with R_F 0.49. The mixture was treated with triethylamine, filtered, and concentrated. Column chromatography (hexane/ethyl acetate 1:1 v/v) of the residue afforded **7** (170 mg, 76.1 %) as a syrup: $[\alpha]_D$ -12° (*c* 1.0, chloroform); R_F 0.49 (hexane/ethyl acetate, 1:1 v/v); ^{1}H NMR ($CDCl_3$) δ 7.30-7.50 (m, 20H, Ph), 5.53 (dd, 1H, H-2"), 5.08 (d, 1H, H-1'), 4.98 (d, 1H, H-1"), 4.91 (d, 1H, H-1), 4.88 (dd, 1H, H-2"), 4.09 (dd, 1H, H-2'), 4.00 (dd, 1H, H-2), 3.82-3.95 (m, 3H, H-3,3',3"), 3.75 (m, 3H, H-5,5',5"), 3.42 (m, 3H, H-4,4',4"), 2.20 (s, 3H, Ac), 1.45 (d, 9H, H-6,6',6"); ^{13}C NMR ($CDCl_3$) δ 102.6 (C-dioxolan), 100.3, 99.1 and 98.9 (C-1,1',1").

5-(1,3-Dioxolan-2-yl)-3-oxabutyl 3,4-Di-*O*-acetyl-2-*O*-[3,4-di-*O*-acetyl-2-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl]-α-L-rhamnopyranoside (8). A solution of **7** (100 mg, 0.085 mmol) in ethyl acetate-methanol (4 mL, 1:1 v/v) was stirred in the presence of 5 % Pd/C (100 mg) under hydrogen. After 24 h, TLC (ethyl acetate/methanol 1:1 v/v) indicated the disappearance of the starting material at R_F 0.6 and a new spot, absent in UV light, at R_F 0. The mixture was filtered through a short column of Celite, concentrated, and the residue was dissolved in pyridine (3 mL). To this solution was added acetic anhydride (3 mL) and the mixture was stirred for 6 h. Concentration and coconcentration with toluene (3 x 1 mL) afforded (72 mg, 96 %) as a syrup:

$[\alpha]_D$ -18° (*c* 1.0, chloroform); R_F 0.34 (hexane/ethyl acetate 1:1 v/v); ^{1}H NMR ($CDCl_3$) δ 5.36 (dd, 1H, H-3"), 5.30 (dd, 1H, H-2"), 5.23 (m, 2H, H-3,3'), 5.01-5.16 (m, 3H, H-4,4',4"), 4.92 (d, 1H, H-1'), 4.85 (d, 1H, H-1"), 4.80 (d, 1H, H-1), 4.16 (dd, 1H, H-2'), 4.07 (dd, 1H, H-2), 3.80-4.00 (m, 3H, H-5,5',5"), 2.01, 2.05, 2.06, 2.11, 2.15 and 2.28 (6s, 21H, 7Ac), 1.10-1.30 (m, 9H, H-6,6',6"); ^{13}C NMR ($CDCl_3$) δ 102.6 (C-dioxolan), 100.2 (C-1), 99.3 (C-1"), 98.6 (C-1'), 77.5 (C-2'), 77.0 (C-2), 71.0 and 71.4 (C-4,4',4"), 70.6 (C-3'), 70.1 (C-3), 69.8 (C-2"), 68.6 (C-3"), 66.3, 66.9 and 67.1 (C-5,5',5"), 17.2 and 17.4 (C-6,6',6").

Anal. Calcd for $C_{38}H_{56}O_{23}$: C, 51.83; H, 6.41. Found: C, 51.45; H, 6.30.

Ring-opening of a Dioxolane and Deacetylation (10). A solution of **8** (72 mg, 0.08 mmol) in formic acid (1 mL) was stirred at room temperature. After 2 h, TLC (dichloromethane/acetone 6:1 v/v, aniline phthalate) indicated the disappearance of the starting material and a new spot at R_F 0.56. The acid was removed with nitrogen gas at room temperature, and toluene was coevaporated several times from the residue: ^{1}H NMR ($CDCl_3$) δ 9.73 (HC=O); ^{13}C NMR ($CDCl_3$) δ 100.2 (C-1), 99.3 (C-1"), 98.6 (C-1').

A solution of sodium methoxide in anhydrous methanol (0.1 mol/L) was added at 0 °C to a solution of the residue (**9**) in dry methanol (0.3 mL) until pH 9. After 45 min, TLC (ethyl acetate/methanol/water 5:5:1 v/v) revealed complete deacetylation of the starting material. The mixture was neutralized with acetic acid, concentrated at room temperature and used directly for conjugation. Yield 38.6 mg (91 %).

Conjugation with Proteins. The free trisaccharide **10** was quantified by the phenol-sulfuric acid method,[14] using rhamnose as standard. The trisaccharide (10 mg) was conjugated with BSA or KLH in a 0.2 M borate buffer, pH 9 at 50 °C for 36 h using sodium cyanoborohydride according to the following molar proportion: 112/1/560 for BSA and 1000/1/5000 for KLH. The resulting solution was dialysed affording BSA-trisaccharide$_{20}$ and KLH-trisaccharide$_{336}$.

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